VERIFICATION FOR TRANSLATION

I, Norio SAEKI, a national of Japan, c/o SAEKI & PARTNERS of 9th Floor, Taka-ai Building, 15-2, Nihonbashi 3-chome, Chuo-ku, Tokyo 103-0027, Japan, do hereby solemnly and sincerely declare:

1) THAT I am well acquainted with the Japanese language, English language, and

2) THAT the attached is a true, accurated and faithful translation into the English language made by me of Japanese Patent Application No. 10-242596/1998 filed to the Japanese Patent Office on August 14, 1998.

Signed this 7th day of October , 2004 .

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[Name of Document] Drawings 1

[Name of Document] Abstract 1

[Document]Specification

[Title of Invention] NUCLEIC ACID CAPABLE OF BINDING SPECIFICALLY TO Ras TARGET PROTEIN

[Claims]

[Claim 1] A nucleic acid capable of being specifically bound to a target protein of Ras.

[Claim 2] The nucleic acid as claimed in claim 1, which nucleic acid is an RNA.

[Claim 3] The nucleic acid as claimed in claim 1 or 2, which nucleic acid is specifically bound to a Ras binding domain of the target protein of Ras.

[Claim 4] The nucleic acid as claimed in any of claims 1 to 3, wherein the target protein of Ras is Raf-1.

[Claim 5] An RNA having any one of base sequences of sequence Nos. 1 to 24 of Sequence Listing.

[Claim 6] The RNA as claimed in claim 5, which RNA having any one of base sequences of sequence Nos. 1 to 8 of Sequence Listing.

[Claim 7] An RNA having any one of base sequences of sequence Nos. 25 to 48 of Sequence Listing.

[Claim 8] The RNA as claimed in claim 7, which RNA having any one of base sequences of sequence Nos. 25 to 32 of Sequence Listing.

[Claim 9] The RNA as claimed in any one of claims 5 to 8, which RNA has specific binding ability to Raf-1.

[Claim 10]The RNA as claimed in claim 9, which RNA is an RNA that is specifically bound to a Ras binding domain (RBD) of Raf-1.

[Claim 11]The RNA as claimed in claims 9 or 10, wherein the RNA is an RNA containing at least any one of base sequences of sequence Nos. 1 to 48 of Sequence Listing or a base sequence in which at least one base thereof is deleted and substituted with another base and/or at least one base is added.

[Claim 12]A DNA having a complementary base sequence to the RNA as claimed in any one of claims 5 to 11.

[Claim 13] An agent for controlling cell signal transduction comprising the nucleic acid as claimed in any of claims 1 to 12.

[Claim 14] The controlling agent as claimed in claim 13, wherein the nucleic acid is an RNA.

[Claim 15] A method of controlling cell signal transduction using the nucleic acid as claimed in any of claims 1 to 12.

[Claim 16]The method as claimed in claim 15, wherein the nucleic acid is an RNA.

[Claim 17] A pharmaceutical composition comprising the nucleic acid as claimed in any of claims 1 to 12.

[Claim 18]The pharmaceutical composition as claimed in claim 17, which composition is used for treating cancers or inflammatory diseases.

[Claim 19]A method of selecting an RNA having an a specific

binding ability to a target protein of Ras, which comprises selecting the RNA having the binding ability to the target protein of Ras from an RNA pool having various base sequences.

[Claim 20]The method as claimed in claim 19, wherein the RNA of the RNA pool having various base sequences is an RNA comprising of 20 to 300 bases.

[Claim 21]The method as claimed in claim 19 or 20, wherein the target protein of Ras is Raf-1.

[Detailed Description of Invention]

[0001]

[Field of the Invention]

The present invention relates to a novel nucleic acid (aptamer) which is specifically bound to target proteins of Ras. More preferably, the present invention relates to a novel RNA aptamer which is specifically bound to Raf-1. Further, the present invention relates to control of signal transduction that induces proliferation or differentiation of cells using the nucleic acid of the present invention, and to a pharmaceutical composition using the same.

[0002]

[Related Art]

Ras is a guanine nucleotide binding protein, and is a protein which participates in signal transduction of cells. When a receptor of cells is activated, GDP binds to Ras protein in cells and becomes "GDP binding Ras". "GDP binding Ras" is further

phosphorylated which becomes "GTP binding Ras".

This "GTP binding Ras" is bound to target proteins of Ras such as Raf-1, B-Raf, RGL, Ral GDS, MEKK, P13K and the like, and transmit necessary signals into cells.

[0003]

These "target proteins of Ras" have a Ras binding domain to which the GTP binding Ras can be bound, and the GTP binding Ras is bound to this domain to transmit necessary signals into cells.

Ras is a key protein of intracellular signal transduction, and the "target proteins of Ras", such as Raf-1, are a center of the intracellular signal transduction system in which signals from Ras are transmitted according to the types.

[0004]

Accordingly, a substance capable of specifically blocking the binding domain with the GTP binding Ras in the "target proteins of Ras", if any, can specifically inhibit an intracellular signal transduction system by Ras, and it is useful to treat or prevent various diseases triggered by the signal transduction. For example, with respect to cancer cells, proliferation or differentiation of cancer cells can be inhibited by specifically controling the signal transduction that induces proliferation or differentiation with the "target cells of Ras" to treat cancers or inhibit metastasis.

[0005]

By the way, Raf-1, one of the "target cells of Ras" is a serine/threonine protein kinase present in a cytoplasm, and the activity is induced by binding to the GTP binding Ras. The activated Raf-1 activates MEK (MAPK/ERK kinase) which is one of the family of mitogen-activated protein kinases, transforming it into phosphorylates extracellular signal-regulated kinase, and thus participates in the signal transduction system (cited reference 1 and 2).

[0006]

In order to elucidate such an intracellular signal transduction system of Raf-1, a method of selectively inhibiting the function of Ras or Raf-1 has been utilized (cited reference 15). These studies include inhibition of the Ras function with a Raf-1 mutant which is free from a kinase activity (cited reference 16), inhibition of a Raf-1 kinase with an antibody bound to a kinase domain of Raf-1 (cited reference 17) and the like.

[0007]

However, these inhibitors do not specifically inhibit a specific part of a signal transduction system with Ras or Raf-1, but inhibit many functions such as a function of binding to Ras, a kinase function and the like simultaneously and diversely. Accordingly, a signal transduction system to be inhibited cannot be specified. Thus, individual specific mechanisms of a signal transduction system could not be clarified satisfactorily.

Consequently, the development of a molecular species capable of specifically inhibiting the binding of Ras to Raf-1 has become important for clarifying the role of the signal transduction system.

[0008]

At present, a downstream signaling pathway of Ras has not been completely clarified. When such a molecular species is developed, it is possible to elucidate the signaling pathway in which Ras participates using a molecular species capable of specifically inhibiting some specific routes and clarify the signaling pathway with target proteins of Ras in detail. In addition, it is possible to control the intracellular signal transduction. Consequently, various diseases in which the intracellular signal transduction participates, such as tumors and the like, can be treated and prevented.

[0009]

Meanwhile, the structural analysis of the "target proteins of Ras" in the intracellular signaling pathway in which Ras participates has been conducted. It has been known that the Ras binding domain (RBD) of Raf-1 is located from 51 to 131 residues in the N-terminus of Raf-1 (cited reference 6 and 18).

[0010]

Further, nucleic acid molecular species (aptamer), such as RNA, DNA and the like, having high affinity for a certain target such as proteins, have been isolated by "in vitro

selection" methods (cited reference 22 and 23) (cited reference 19, 20 and 21).

[0011]

[Problems to be Resolved by the Invention]

The present invention is to provide nucleic acid molecular species which can specifically inhibit the binding to "GTP binding Ras" by being specifically bound to a Ras binding domain (RBD) of target proteins of Ras such as Raf-1, B-Raf, RGL, Ral GDS, MEKK, P13K and the like.

[0012]

The present inventors have found that a nucleic acid molecular species specifically bound to the Ras binding domain of the "target proteins of Ras" can be obtained by using the in vitro selection method. For example, it has been possible to obtain a novel RNA aptamer targeting the Ras binding domain (RBD) of Raf-1, one of the "target proteins of Ras" by this method and to determine the RNA sequence thereof. This RNA aptamer can specifically inhibit the binding between Ras and Raf-1.

[0013]

The present invention is to provide RNA aptamer that bound to a "target proteins of Ras", especially its RBD, and a method of controlling a signal transduction system using such RNA aptamer.

[0014]

[Means of Solving Problems]

The present invention is to provide a nucleic acid which specifically controls the intracellular signal transduction participated by Ras proteins and target proteins of Ras.

The present invention relates to a nucleic acid, preferably RNA, which is capable of being specifically bound to the target proteins of Ras. Specifically, the present invention relates to a nucleic acid, preferably RNA, which is capable of being specifically bound to Ras binding domain (RBD) of target proteins of Ras. More specifically, the present invention relates to a nucleic acid, preferably RNA, which is capable of being specifically bound to target protein of Ras wherein target protein of RAS is Raf-1.

[0015]

Further, present invention relates to RNA having any one of base sequences of sequence Nos. 1 to 24 of Sequence Listing, preferably base sequences of sequence Nos. 1 to 8 of Sequence Listing. The present invention also relate to RNA having any one of base sequences of sequence Nos. 25 to 48 of Sequence Listing, preferably base sequences of sequence Nos. 25 to 32 of Sequence Listing.

The RNAs of the present invention shown in the sequence numbers have an ability of binding to the Raf-1, and more specifically, the RNAs are characterized in that they are specifically bound to the Ras binding domain (RBD) of Raf-1.

[0016]

Therefore, the present invention relates to RNA having a binding ability to Raf-1 comprising a base sequence in which at least one base of sequence Nos. 1 to 48 of Sequence Listing is deleted and substituted with another base and/or another base is added.

These RNAs of the present invention can also be reversely transcribed, as required, into DNAs having complementary base sequences to the RNAs. Accordingly, the present invention relates to nucleic acids such as RNAs, DNAs and the like, containing any one of base sequences of sequence Nos. 1 to 48 of Sequence Listing or a base sequence in which at least one base thereof is deleted and substituted with another base and/or at least one base is added.

[0017]

Further, the present invention relates to agent for controlling cell signal transduction which agent is made of above-mentioned RNA, or the method for controlling cell signal transduction using said RNA.

Furthermore, the present invention relates to a pharmaceutical composition containing the above-mentioned RNA, more specifically it relates to a pharmaceutical composition for diseases in which the cell signal transduction participates such as treatment or prevention of cancer or inflammatory diseases.

[0018]

The "target proteins of Ras" of the present invention refers to Ras proteins which participate in the cell signal transduction, preferably a group of proteins forming an intracellular signal transduction system by interacting with GTP binding Ras proteins. Examples of the "target proteins of Ras" of the present invention include Raf-1, B-Raf, RGL, Ral GDS, MEKK, P13K and the like, however, this invention is not limited within the target. The "target proteins of Ras" of the present invention are preferably Raf-1 and the like.

[0019]

The present invention has clarified that nucleic acid molecular species capable of being specifically bound to the foregoing "target proteins of Ras" exist. Accordingly, the nucleic acid specifically bound to the "target proteins of Ras" in the present invention may be an RNA or a DNA. The RNA or the DNA is not particularly limited so long as it is specifically bound to the "target proteins of Ras". Further, the nucleic acid of the present invention may be specifically bound to only one "target protein of Ras" or to two or more "target proteins of Ras".

[0020]

The size of bases of the nucleic acid molecular species of the present invention is not particularly limited so long as it is sufficient to allow the specific binding to the "target proteins of Ras". It is between 20 and 300 bases, preferably

between 20 and 150 bases, more preferably between 30 and 150 bases, further preferably between 30 and 100 bases. In the case of emphasizing the binding specificity, the longer size is preferable, however, in view of the availability such as by synthesis method or the like, the shorter size is preferable.

[0021]

The "aptamer" in the present invention refers to a nucleic acid molecular species capable of being bound to a specific domain of a protein, and the nucleic acid may be an RNA or a DNA. An aptamer made of an RNA is called an "RNA aptamer".

[0022]

The nucleic acid (aptamer) of the present invention can be produced by various methods. When the base sequence of the aptamer is known, it can be synthesized.

[0023]

When the base sequence of the aptamer of the present invention is unknown, the aptamer can be produced through selection by the known "in vitro selection" method (cited references 22 and 23). The "in vitro selection" method in the present invention is described.

[0024]

First, RNAs containing a random base sequence of 20 to 300 bases, preferably 30 to 100 bases, more preferably 30 to 70 bases are prepared. These RNAs are prepared by transcription from synthetic DNAs containing a random sequence.

A base sequence which is to be a primer in the PCR method is added to the 5'-terminus and the 3'-terminus of the DNAs. In this case, the primer is not particularly limited, however, a primer having a sequence of cleavage with a restriction endonuclease so as to be able to cleave this primer portion later is preferable. A size of the primer portion is not particularly limited. It is approximately between 20 and 50 bases, preferably between 20 and 30 bases. Further, a promoter sequence of a T7 RNA polymerase is added to the primer at the 5'-terminus, enabling the transcription reaction from DNA to RNA.

[0025]

In this manner, the RNA group (RNA pool) having the base sequences as the primer at both termini and the random base sequence in the center is prepared by transcription of the DNA.

Subsequently, the RNA in this RNA pool and the "target protein of Ras", for example, Raf-1 or a peptide comprising its binding domain are contacted to separate the RNA bound to the "target protein of Ras". The selected RNA is converted to a cDNA through reverse transcription, and it is amplified by PCR using the primers. The DNA amplified is transcribed into an RNA, and this is returned to the RNA pool.

[0026]

One cycle, termed a "round", comprises binding with the "target protein" of Ras in the RNA pool, the separation of the bound RNA, reverse transcription, amplification by PCR and

transcription of the DNA. That is, one round means that the foregoing round is conducted once.

[0027]

When the foregoing round using the RNA pool is conducted, the amount of the RNA bound to the "target protein of Ras" in the RNA pool is increased, and further the amount of the RNA having the specific binding base sequence is increased, so that the RNA to be specifically bound can be selected by repeating the round.

Such a round is conducted 5 to 50 times, preferably 5 to 30 times.

[0028]

The RNA sequences selected by the "in vitro selection" method as described above are determined by a usual method, and this RNA can also be converted to a cDNA through reverse transcription by a usual method. Further, the primer regions can be cleaved as required.

In this manner, the aptamers of the present invention can be obtained.

[0029]

The "in vitro selection" method of the present invention is described in more detail by using Raf-1 as the "target protein of Ras".

[0030]

The present inventors have prepared an RNA pool having a

random sequence of approximately 60 bases to select RNAs bound to the Ras binding domain (RBD) of Raf-1. And, the base sequences shown in Fig. 1 were bound to the 3'-terminus and the 5'-terminus of these RNAs.

[0031]

It was presumed that approximately 8×10^{13} sequences of RNAs are present in this RNA pool.

Before conducting the selection of the aptamer, the effect of the salt concentration relative to the binding between the RNA pool and Raf-1-RBD was examined. At a low salt concentration, the RNA was non-specifically bound to Raf-1-RBD. However, it was found that the non-specific binding is suppressed by increasing the salt concentration (up to approximately 150 mM). Thus, the present inventors used a phosphate buffer (hereinafter referred to as a "binding buffer") containing 137 mM sodium chloride as a buffer for the selection.

[0032]

The selection from the 1st to 13th rounds was conducted by the binding between a fusion protein of glutathione S-transferase (GST) and a peptide (RBD) of 51 to 131 of Raf-1 (hereinafter referred to as "GST-RBD") and RNAs using a glutathione-Sepharose 4B matrix. In the 13th round, the binding ability (binding ratio) to the RNA pool was slightly increased from 0.16% at the initial stage to 0.36%.

Successively, the selection using a nitrocellulose filter

instead of the matrix was conducted eight times (8 rounds). In the 21st round, the binding ability to the RNA pool was 22%, and the Kd value of the pool relative to the protein GST-RBD was 290 nM.

[0033]

The sequences of 33 clones were determined from the RNA pool which finished the 21st round. Consequently, 24 different sequences were obtained. These 24 different sequences are shown in Fig. 2. They were roughly divided into two types, one having a high homology among the sequences (this group is called "group 1") and the other among which no homology, was observed (these are referred to as "group 2").

The sequences of the total sizes (approximately 100 bases) of the 8 types of RNAs (21.01 to 21.08 in Fig. 2) in group 1 are shown in sequence Nos. 1 to 8.

[0034]

The interaction between the 10 RNAs among them and GST-RBD was examined by a binding assay using a nitrocellulose filter (refer to Fig. 2). As a result, the RNAs in group 1 showed the satisfactory binding to GST-RBD, whereas the RNAs in group 2 did not show the satisfactory binding. The Kd values of the RNAs shown in sequence Nos. 1 (21.01 in Fig. 2) and 7 (21.07 in Fig. 2) were both 300 nM. Meanwhile, that of the RNA in sequence No. 11 (21.11 in Fig. 2) was of micromol unit (refer to Fig. 3). Fig. 3 shows percentages of binding to GST-RBD when using RNAs

having sequence Nos. 1 (black circle), 7 (black square) and 11 (black triangle) at various concentrations (nM).

[0035]

Further, the RNA ligands were not bound to GST itself. This indicates that these RNA is bound to the RBD moiety of GST-RBD rather than to the GST moiety.

[0036]

It was then examined whether the RNA aptamers in group 1 inhibit the interaction between Ras and RBD (refer to Fig. 4). The RNAs having sequence Nos. 1 (A in Fig. 4), 7 (B in Fig. 4), 11 (C in Fig. 4) and 12 (D in Fig. 4) (21.01, 21.07, 21.11 and 21.12 in Fig. 2) were tested at concentrations of 0 to 12.5 μm. These were incubated with GST-RBD supported on a Sepharose matrix and Ras in GTPγS or GDP. In the presence of the RNAs (lanes 3, 4 and 5 in Fig. 4; lane 3 is 20-pmol RNA, lane 4 200-pmol RNA and lane 5 2,000-pmol RNA) or in the absence of the RNAs (lanes 1 and 3 in Fig. 4; lane 1 was in the presence of GDP and lane 2 in the presence of GTP), the binding between GST-RBD and Ras was examined by immunoblotting with anti-Ras antibody RAS004.

In Fig. 4, "Ras" indicates Ras bound to GST-RBD, and "GST-RBD" indicates as a background that GST-RBD is solely present.

[0037]

As stated earlier, the RNA of sequence No. 12 which is scarcely bound to GST-RBD did not inhibit the binding of Ras

to GST-RBD even at the concentration of 12.5 μM. This was the same with the RNA of sequence No. 11 in which the kd value was a micromol order.

[0038]

On the other hand, the RNAs of sequence Nos. 1 and 7 in group 1 effectively inhibited the interaction between Ras and RBD. The reason is considered to be that these RNAs were bound to RBD. And, the kd value of the GTP binding Ras and RBD of Raf-1 is 18 nM (cited reference 30), and the RNAs of sequence Nos. 1 and 7 have the binding ability which is 10 times lower than that. Despite this, these RNAs inhibit the interaction between Ras and Raf-1.

[0039]

Since these RNAs do not have an affinity for Ras or a Sepharose matrix, there is no possibility that these RNAs are bound to Ras or the Sepharose matrix to inhibit the binding between GST-RBD and Ras on the matrix. This fact proved the specific binding of these RNA aptamers to RBD.

[0040]

An antibody bound to Raf-1 mutant which is free from a kinase activity or a kinase domain of Raf-1 has been used to study the role of Ras or Raf-1 in the cell signal transduction system (cited references 16 and 17).

The Raf-1 mutant capable of being bound to Ras without having the kinase activity not only inhibits the Ras-dependent Raf-1

activity but also blocks the wide-ranging signal transduction systems including Ras. This is because the mutant inhibits the binding of Raf-1 and also has an influence on various downstream effectors of GTP-binding Ras.

[0041]

Likewise, a monoclonal antibody bound to an epitope of a kinase domain of Raf-1 inhibits all signal transduction systems including Raf-1. This is because Raf-1 is activated not only with GTP binding Ras but also through a route having no bearing on Ras (cited reference 17).

[0042]

From this standpoint as well, it is said that the RNA aptamer to RBD in the present invention can specifically inhibit the binding between Ras and Raf-1 without having any effect on the kinase activity of Ras or Raf-1 by the other signaling pathways.

Further, the RNA aptamers of the present invention can be expressed within cells (cited reference 31), and can be applied to a wide-ranging field. For example, in case of treatment for human diseases, it may be applied to treatment for inflammation (cited reference 32) and RNA treatment for pneumonia (cited reference 33).

[0043]

Thus, the RNA aptamers of the present invention specifically block RBD of "target proteins of Ras", more preferably Raf-1. Not only it can be used in an agent for controlling intracellular

signal transduction, but also it is especially suited for the field of treatment, prevention or diagnosis of various diseases in which the signal transduction system participates.

[0044]

When the nucleic acids of the present invention are used in controlling the cell signal transduction system, the nucleic acids of the present invention may directly be introduced into desired cells. It can also be introduced into cells by being inserted into viruses or the like.

Further, it is also possible that the RNA is introduced not directly but in the form of a DNA.

[0045]

When the nucleic acid of the present invention is used as a pharmaceutical composition, it can parenterally be administered as such, or it can be administered by being inserted into viruses or various vectors in the form of a DNA. In these administration forms, the pharmaceutical composition can also be provided using a pharmaceutically acceptable carrier.

The pharmaceutical composition of the present invention is useful for treatment, prevention or diagnosis of various diseases in which the cell signal transduction system participates, especially malignant tumors and inflammatory diseases.

[0046]

[Example]

The present invention is illustrated more specifically with reference to the following Examples. However, the present invention is not limited to these Examples.

[0047]

Example 1 (Purification of a protein)

Glutathione S-transferase (GST) and a fusion protein of RBD (51 to 131 amino acid moiety of Raf-1) and GST (hereinafter referred to as GST-RBD) were expressed in E. coli strains BL21 and BL21DE3 respectively, and purified by chromatography using a column of glutathione-Sepharose 4B (made by Amersham Pharmacia Biotec) and HQ proth (perceptive) (cited reference 24).

[0048]

As wild type Ha-Ras protein, a protein obtained from E. coli strain BL21 was purified by column chromatography using DEAE-Sephacell, Sephadex G-75 and Resource QFPLC (made by Amersham Pharmacia Biotec)(cited references 24, 25 and 26).

The purity of these proteins were identified through SDS-PAGE by staining with Coomassie Blue and/or silver. The purified protein was stored in 50% glycerol at -30°C.

[0049]

Example 2 (In vitro selection)

A pool of DNAs containing random 60 bases was prepared. These DNAs have sequences 5'-GCCGGAATTCTAATACGACTCACTATAGGGAGATCAGAATAAACGCTCAA-3' and 5'-CGCCCTGCAGGGGCCTCATGTCGAA-3' at both termini for in vitro

transcription and amplification by PCR.

These RNAs were heated at 75°C for 3 minutes, then ice-cooled, and incubated in a binding buffer (5 mM $MgCl_2$-containing phosphate buffer physiological saline solution) containing GST-RBD and glutathione-Sepharose 4B beads. The RNAs having GST-RBD bound thereto were recovered with glutathione-Sepharose beads. The beads were cleaned with a cleaning buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$ and 150 mM NaCl), and the RNAs on the beads were eluted with boiling water.

[0050]

The 1st through 13th rounds were conducted in this manner, provided that in order to remove the RNAs bound to GST and/or the beads alone, the RNAs were passed through glutathione-Sepharose beads having supported thereon GST only before the incubation of GST-RBD (3rd to 13th rounds) and after the elution (7th to 13th rounds).

The eluate was subjected to reverse transcription, and amplified through PCR. The RNA pool for the next round was prepared through in vitro transcription from the cDNA amplified. The 13th round and those following for selection were conducted by a filter binding method. The RNA pool (heated at 75°C for 3 minutes, and cooled to room temperature) and GST-RBD were incubated in a binding buffer at 37°C for 1 hour. The RNAs bound to GST-RBD were separated by being bound to a nitrocellulose filter. The RNAs were eluted with a buffer containing 7 M urea

(cited reference 27). In order to remove the RNAs bound to the filter, the RNAs were passed through the filter before the amplification.

[0051]

Example 3 (Nitrocellulose filter binding assay)

RNAs were transcribed in vitro with a T7 RNA polymerase using [α-^{32}P]UTP. The RNA (0.8 μM) and its protein were incubated in 50 μl of a binding buffer at 37°C for 1 hour. A part (50 μl) of the solution was moved on a filter, and cleaned three times with 200 μl of a cleaning buffer.

[0052]

In order to determine a dissociation constant, 1.6 nM of the RNA of which the 5'-terminus was labeled with [γ-^{32}P]ATP and GST-RBD at various concentrations were incubated. The radiation dose on the filter was measured using a Fuji BAS2500 bio-imaging analyzer.

[0053]

Example 4 (Inhibitory activity)

One microgram of GST-RBD in 160 μl of a binding buffer containing 0.05% Triton X-100 was mixed with 10 μl of a glutathione-Sepharose bead suspension in a phosphate buffer physiological saline solution. The mixture was incubated at 4°C for 30 minutes. After the gentle centrifugation, the supernatant liquid was discarded. A binding buffer solution containing 40 ng of Ras (this is bound through GTPγS or GDP as

described in a literature (cited reference 3) and 160 μl of the RNA was added to the remaining beads, and incubated at 4°C for 30 minutes. After the incubation, the beads were cleaned with 500 μl of a cleaning buffer. The bound protein was eluted from the beads through separation using a Laemmli's buffer, and subjected to 15% SDS-PAGE. The product was subjected to immunoblotting with anti-Ras antibody RAS004 (cited reference 4), and visualized with an ECL immune detector (manufactured by Amersham Pharmacia Biotec).

[0054]

[Effects of the Invention]

The present invention provides RNAs which are specifically bound to target proteins of Ras such as Raf-1 and the like and which further inhibit the binding to Ras, and a method of specifically inhibiting an intracellular signaling pathway using these RNAs. The present invention can not only clarify the signaling pathway through the specific route of cells but also provide a pharmaceutical composition having less side effects.

[Sequence Listing]

SEQUENCE LISTING

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[Brief Description of the Drawings]

[Figure 1]

Fig. 1 shows a sequence of an initial RNA pool in the in vitro selection of the present invention and sequences of PCR primers.

[Figure 2]

Fig. 2 shows 24 sequences of RNAs obtained from the RNA pool in the 21st round. In the drawing, a random sequence moiety of approximately 60 bases is shown. The overall sequence includes the sequences of the 5'- and 3'-termini defined in Fig. 1. Sequences which are the same as that of clone 1 are termed "group 1". The other sequences are termed "group 2".

Annotation a: The number of clones of ligands isolated respectively is shown in parentheses.

b: Clone 21.08 indicates that each sequence defined has two mutations.

c: Percentage of the binding of the RNA ligand to GST-RBD is based on a value measured by the nitrocellulose filter binding assay.

[Figure 3]

Fig. 3 shows the binding of the RNA ligand to the GST-RBD protein. Percentage of the binding of the RNA ligand to GST-RBD is based on a value measured by the nitrocellulose filter binding assay. In Fig. 3, a black circle indicates the use of an RNA of sequence No. 1, a black square the use of an RNA of sequence

No. 7, and a black triangle the use of an RNA of sequence No. 11.

[Figure 4]

Fig. 4 is a photo that replaces a drawing, showing inhibition of interaction between Ras and GST-RBD by the RNA aptamers. An amount of the Ras protein bound to GST-RBD as measured by immunoblotting with anti-Ras antibody RAS004 is shown on an upper column. An total amount of GST-RBD measured by staining with Coomassie Blue is shown on a lower column.

GDP binding (D) or GTPγS binding (T) Ras (2 pmols) and GST-RBD (25 pmols) were incubated in the presence of an RNA in various amounts. A used 21.01 ligand, B 21.07 ligand, C 21.11 ligand, and D 21.12 ligand.

[Document Name]Drawings

[Figure 1]

Starting RNA:
5′-GGGAGAUCAGAAUAAACGCUCAA[-60N-]UUCGACAUGAGGCCCCUGCAGGGCG-3′

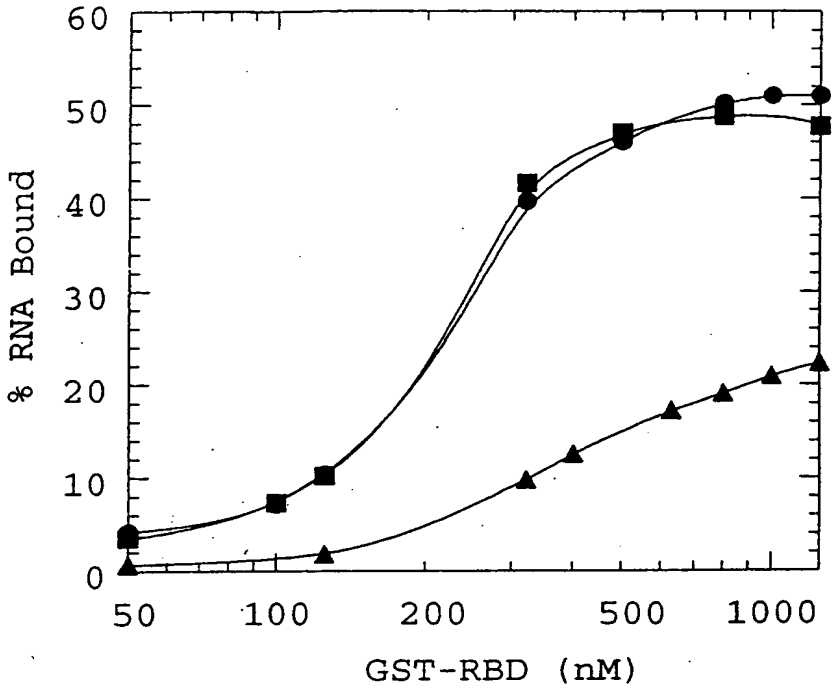
PCR primer 1:
5′-GCCGGAATTC**TAATACGACTCACTATA**GGGAGATCAGAATAAACGCTCAA-3′
EcoRI **T7 promoter**

PCR primer 2:
5′-CGCCCTGCAGGGGCCTCATGTCGAA-3′
PstI

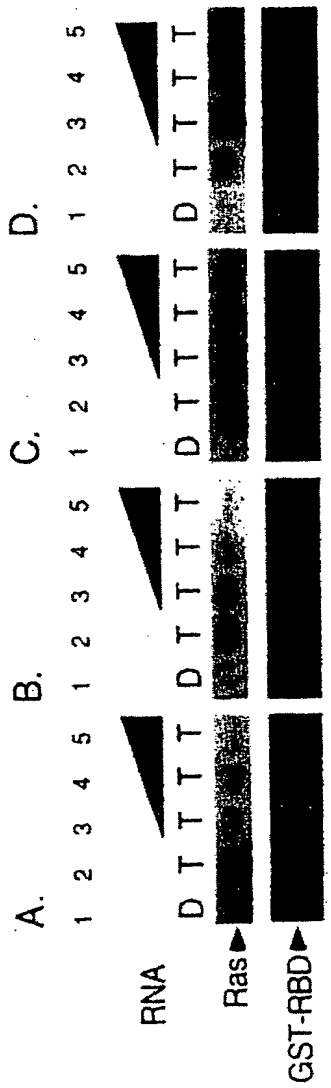
[Figure 21]

RNA clone[a]		Sequence[b]	RNA Bound(%)[c]
Group 1			
21.01	(6)	CUGAUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACCCCUUGGACUGA	42
21.02		CUGAUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACCCC-UGGACUGA	
21.03	(4)	CUGAUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACCCCUUGGACUGC	
21.04		CUGAUCAAUGGCGUACAAUGGAUUCGCUCUCAUAACCAAAACCCUUACCCCUUGGACUGC	
21.05		CUGAUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACUCCUUGGACUGC	46
21.06		CUGAUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACCCCUUGGACUGU	
21.07		UUGACUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACCCCUUGGACUG	46
21.08		UUGAAGAUCGUACAAUGGAUUCG--AUCAUAACCCGAAGUUUUUAAACACUCUUUACCUGUA	12
Group 2			
21.09		UCGAGUCCACGAACAUUACAUAUUUGAACACUUCAGCACCGAACAUGCUUAGUACUAUCC	
21.10		UAUUACCAUAGCCUUGAGGUAAACAAUUUAGCACACCUGAAUACACGAACUAUGAACUCA	
21.11	(2)	CUUGAGCCAAUUAAAAGAUUUACAACAAGAACAUGAACGUGACAGCGAUAAUAAUACGA	3
21.12		GCGACAAGCAGCAGAUAAAGUUGAGCGCAACGCCGCUACAGAACCAAAUUAACAUGUAUG	0.2
21.13		UCGAAAGUAAGUCCGAUACAACACAUAACCUAUUAUUUAGCAGCGAUAAUACAAAUAAG	
21.14		GCAGUAAUCCACUUGUAAUUGAAUGUAGAUGCCAUAUAGAGUUAUUAGUAAUCCGAAUUG	
21.15		CGUAGUAGCACACCAUGACCUAUUAAAUCUGCUUCGCAAUGUACCUUAACACAUAAUCAG	
21.16		GAAUGACUAAUAAUUACAACAGAUAACCUUACUCUUGAUAAAUGCUUUGCUUUUGGUUAA	1
21.17		UCUUCGAAGUCCAUGACUGCAAAACCAGAUAGUCCUAAUCUCAAUUAUCAGUCCCAAGUA	
21.18		ACACUCUAAAUUGUGGUACUAAGGGAGUAAGGGCAACUACGAAGACGUGCAAGGAUAAAG	0.5
21.19		UUUGCCUCGACGGUCUGCGAAUAGAACGCGAACCGUGAUUAGUGUACAAGGAUUCGGUU	
21.20		GUCGCAGCAGAAAUAUCAUCGCAAAACCUCAAUUGCAUCUCAUGUAUAUCUAGUCCAA	0.7
21.21		CGAACAUCUGGAGUAAUCAUCUUAAUAACCUCAUUAACCUUUACACUUUCUAAACUA	
21.22		GGGUAAGGGUGAGCAGUUCAAGAUGGUAACUGGCAUUCAUUUGAAGAAAGGUUGGUAGAC	1.3
21.23		GGGUAAGGGUGAGCAGUUCAAGAUGGUAACCGGCAUUCAUUUGAAGAAAGGUUGGUAAAC	
21.24		CUUGGUGUAGUGUUCAAGUGAGAUAUAGUAUAAGGUUAUUGUUGUGCGAACGG	

[Figure 3]



[Figure 4]



[Document] Abstract

[Abstract]

[Problem]

Present invention provides a nucleic acid which specifically controls the intracellular signal transduction participated by Ras proteins and target proteins of Ras.

[Means for Solving Problem]

Present invention relates to nucleic acid, preferably RNA, which is capable of being specifically bound to the target proteins of Ras.

[Chosen Drawing] None